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Research article

## Possible mechanism(s) for relaxant effect of aqueous and macerated extracts from *Nigella sativa* on tracheal chains of guinea pig

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Published: 25 February 2004

Received: 29 November 2003

BMC Pharmacology 2004, 4:3

Accepted: 25 February 2004

This article is available from: <http://www.biomedcentral.com/1471-2210/4/3>

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### Abstract

**Background:** In previous studies, the relaxant, anticholinergic (functional antagonism) and antihistaminic effects of *Nigella sativa* have been demonstrated on guinea pig tracheal chains. To elucidate the other mechanisms responsible for the relaxant effect of this plant, its inhibitory effect on the calcium channel was examined in this study.

**Results:** The inhibitory effects of both concentrations of diltiazem in all three groups of experiments were significantly greater than those of saline ( $p < 0.01$  to  $P < 0.001$ ). The inhibitory of two larger concentrations of aqueous extracts in group 1 and 2 were significantly greater than those of saline ( $p < 0.01$  to  $P < 0.001$ ). The effect of two larger concentrations of macerated extract in group 1 and all concentrations of this extract in group 2 were also significantly greater than those of saline ( $p < 0.01$  to  $P < 0.001$ ). However, the extract of *Nigella sativa* did not show any inhibitory effect in group 3. There was a significant correlation between inhibitory effect and increasing concentrations for both extracts and diltiazem in groups 1 and 2 ( $p < 0.05$  to  $p < 0.005$ ).

**Conclusion:** Although the extracts of *Nigella sativa* showed inhibitory effects on pre-contracted tracheal chains in the presence of both ordinary and calcium free Krebs solution, the absence of inhibitory effects of the extracts on KCl induced contraction of tracheal chains suggest that the calcium channel blocking effect of this plant dose not contribute to the relaxant effect of this plant on the tracheal chains of guinea pigs.

### Background

*Nigella sativa* L. (Ranunculaceae) is a grassy plant with green to blue flowers and small black seeds, which grows in temperate and cold climate areas. The seeds of *Nigella sativa* contain thymoquinone, monotropens such as *p*-

cymene and  $\alpha$ -pinene [1], Nigellidine [2], Nigellimine [3] and a saponin [4].

Several therapeutic effects including those on digestive disorders, gynaecology, and also anti-asthma and dyspnea

have been described for the seeds of *Nigella sativa* in ancient Iranian medical books [5]. *Nigella sativa* has long been known for its medical use as an antispasmodic, especially against gastrointestinal disorders or respiratory ailments, in many countries.

There is evidence of relaxant effects of volatile oil from this plant on different smooth muscle including rabbit aorta [6], rabbit jejunum [7], and isolated tracheal muscles of guinea pigs [8]. Mahfous and El-Dakhkhny (1960) reported that the volatile oil from *Nigella sativa* protected guinea pigs against histamine-induced bronchospasm, but it did not affect histamine  $H_1$  receptors in isolated tissues [9]. However, in an *in vivo* study, increasing respiratory rate and intra tracheal pressure of guinea pigs due to i.v. administration of volatile oil from *Nigella sativa* has been demonstrated [10]. In our recent studies, a relaxant effect for this plant was shown [11]. In addition the anticholinergic, and histamine  $H_1$  receptor blocking effects of this plant on isolated guinea pig tracheal chains were demonstrated by performing concentration response curves to methacoline and histamine in the presence of saline and plant extracts [11,12]. In both studies the plant extracts caused rightward shift in concentration response curves to methacoline and histamine.

To elucidate the other mechanism(s) responsible for the observed bronchodilatory effect of *Nigella sativa*, the inhibitory effect of aqueous and macerated extracts of this plant on the calcium channel of guinea pig tracheal chains was examined in this study.

## Results

### Inhibitory effect

In group 1 experiments the inhibitory effects of 5  $\mu$ M concentration of diltiazem and 0.5 g% and 1 g% concentrations of both extracts were significantly greater than that of saline ( $p < 0.05$  to  $p < 0.001$ , Table 1). In group 2 experiments the inhibitory effect of both concentrations of diltiazem, all concentrations of macerated extract and 0.5 g% and 1 g% concentrations of aqueous extracts were significantly greater than the effect of saline ( $p < 0.5$  to  $p < 0.001$ , Table 1). In group 3 experiments only both concentrations of diltiazem showed significant inhibitory effect compared to saline ( $p < 0.001$  for both cases, Table 1).

### Differences between the effect of diltiazem and extracts

There was no significant difference between the effects of extracts from *Nigella sativa* with those of diltiazem in group 1 and 2 experiments. However, the effects of all concentrations of aqueous and macerated extracts were significantly lower than those of diltiazem in group 3 ( $p < 0.001$  for all cases).

### Differences of the inhibitory effects between three groups of experiments

The inhibitory effects of all concentrations of macerated extract in group 2 and those of diltiazem in groups 2 and 3 were significantly greater than the effects obtained in group 1 ( $p < 0.05$  to  $p < 0.001$ , Fig 1). The inhibitory effects of all concentrations of aqueous extract in group 2 were also non-significantly greater than those of group 1 (Fig 1). However, the inhibitory effects of all concentrations of extracts in group 3 were significantly lower than those of group 1 and 2 experiments ( $p < 0.001$  for all cases, Fig 1).

### Relationship between concentrations and inhibitory effect

In group 1, only the inhibitory effect of 0.25 g% vs 1 g% aqueous extract was statistically significant ( $p < 0.05$ ). However, in group 2, the differences in inhibitory effects between all concentrations of two extracts except that of 0.5 g% vs 1 g% macerated extract were significant ( $p < 0.05$  to  $p < 0.001$ ). The inhibitory effects of two concentrations of diltiazem were also significantly different in group 2 and 3 ( $p < 0.05$  and  $p < 0.001$  respectively). In addition there were significant correlations between increasing concentrations and inhibitory effects for both extracts in group 1 and 2 experiments ( $p < 0.05$  to  $p < 0.005$ , Table 2).

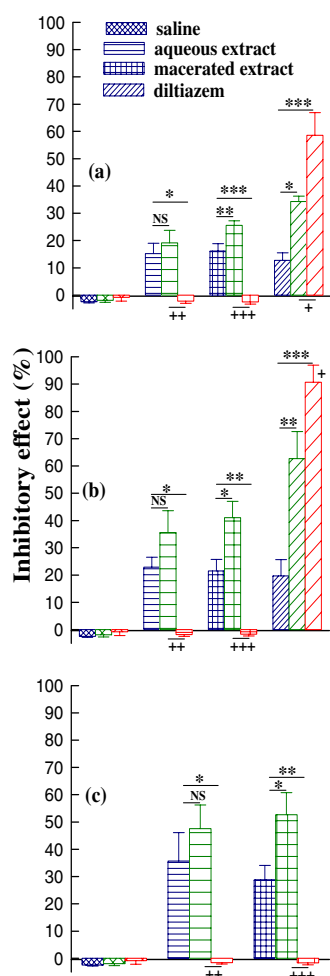
## Discussion

The bronchodilatory effect seen for *Nigella sativa* in our previous study [11] might be produced due to several different mechanisms. With regard to bronchodilatory effect of calcium channel blockers [13,14], one possible mechanism responsible for the bronchodilatory effect of this plant could be the inhibitory effect of this plant on calcium channels. This effect of the aqueous and macerated extracts from *Nigella sativa* was therefore examined on isolated guinea pig tracheal preparations in the present study.

In group 1 experiments (contracted tracheal chains by 10  $\mu$ M methacholine in the presence of ordinary Krebs solution) both extracts and diltiazem showed concentration-dependent inhibitory effects compared with that of saline.

To examine whether the inhibitory effects of extracts seen in group 1 were due to inhibitory effect on calcium channels or not, the inhibitory effects of extracts and diltiazem were re-examined on contracted tracheal chains in the presence of calcium-free Krebs solution in group 2. The results of this group also showed concentration-dependent inhibitory effects of both extracts and diltiazem on methacholine-induced contraction of tracheal chains.

With regard to the inhibitory effect of KCl on calcium channels [15], the inhibitory effects of different



**Figure 1**

Inhibitory effect of different concentrations of aqueous and macerated extracts from *Nigella sativa*, diltiazem, and saline on pre-contracted tracheal chains of guinea pigs. group 1 (methacholine induced contraction of guinea pig tracheal chains in the presence of ordinary Krebs solution, blue colour), group 2 (methacholine induced contraction of guinea pig tracheal chains in the presence of calcium free Krebs solution, green colour) and group 3 (KCl induced contraction of guinea pig tracheal chains in the presence of ordinary Krebs solution, red colour). (a) low concentration, (b) medium concentration, and (c) high concentration, for diltiazem only 2 concentrations (low and medium) were examined. Statistical differences between the results of group 1 with those of group 2 and 3; NS: non significant difference \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Statistical differences between the results of group 2 with those of group 3, +:  $p < 0.05$ , ++:  $p < 0.01$ , +++:  $p < 0.001$ . Aqueous and macerated extracts did not show any inhibitory effect in group 3 experiments compared to the effect of saline.

concentrations of extracts and diltiazem were also examined on KCl induced contraction of tracheal chains (group 3 experiments). In this group of experiments extracts from *Nigella sativa* did not show any inhibitory effect on KCl induced contraction. However, diltiazem showed concentration-dependent inhibitory effect on KCl induced contraction of tracheal chains.

Although, the results of group 1, and mainly group 2, may indicate an inhibitory effect of the extracts from *Nigella sativa* as claimed in a previous study [16]. However, the results of group 3 clearly showed that the extracts from this plant have no inhibitory effects on calcium channels of guinea pig tracheal chains. The cause for methacholine induced contraction of tracheal chains in group 2 is unclear to us, but the most probable explanation is the role of stored calcium in smooth muscle cytoplasm in this phenomenon. A previous study also showed contraction of tracheal smooth muscle in the presence of calcium-free Krebs solution [16]. The mechanism of inhibitory effect of diltiazem on methacholine induced contraction of tracheal chains in the presence of calcium free buffer (group 2) is perhaps due to inhibition of increase in sarcoplasmic calcium concentration released from sarcoplasmic reticulum through IP3 induced calcium release [17].

In our previous study a rightward shift of concentration-response curves of  $\text{CaCl}_2$  in tracheal chains of guinea pigs has been demonstrated for aqueous extract of *Nigella sativa*, indicating a calcium channel inhibitory effect of this extract [18]. However, the results of the present study showed that aqueous and macerated extracts have no inhibitory effect on calcium channels. The cause of rightward shift in  $\text{CaCl}_2$  concentration-response curves of our previous study [18] may be due to the relaxant effect of this plant on tracheal chains.

While KCl affects calcium channels [15] and with regard to bronchodilatory effects of calcium channel blockers [13,14], these findings showed the absence of a blocking effect of the extracts from *Nigella sativa* on calcium channels. The absence of obvious relaxant effects of aqueous and macerated extracts from this plant in group 3 and the relatively potent relaxant effect of this extract in groups 1 and 2 experiments may also indicate an opening effect of these extracts on potassium channels because the bronchodilatory effects of potassium channel opening has been demonstrated previously [19]. If the aqueous and macerated extracts from *Nigella sativa* had a potassium channel opening effect, they would not have relaxant effects on tracheal chains contracted by KCl, while they could show relaxant effect when the tracheal chain was contracted by methacholine. In fact, the results of groups 1 and 2 may support this effect of aqueous and macerated extracts. Therefore, the absence of inhibitory effects of

**Table 1: Inhibitory effect of three cumulative concentrations of aqueous and macerated extracts from *Nigella sativa*, two concentrations of diltiazem and saline in three groups of experiments on pre-contracted tracheal chain of guinea pigs.**

Inhibitory effect									
Experimental design		Group 1	St.Dif vs S.	St. Dif vs D	Group 2	St.Dif vs S.	St. Dif vs D.	Group 3	St.Dif vs S. St. Dif vs D.
Slime	1 ml	-2.43 ± 0.90			-1.93 ± 0.74			-0.83 ± 1.35	
Diltiazem	1 µM	12.72 ± 2.80	NS		34.30 ± 2.02	P < 0.01		58.57 ± 8.36	P < 0.001
	5 µM	19.67 ± 6.00	P < 0.05		62.67 ± 9.62	P < 0.001		90.71 ± 6.21	P < 0.001
Aqueous extract	0.25 g%	15.23 ± 3.80	NS	NS	19.16 ± 4.59	NS	NS	-2.24 ± 0.75	NS P < 0.001
	0.5 g%	22.87 ± 3.66	P < 0.05	NS	35.56 ± 7.99	P < 0.01	NS	-1.85 ± 0.60	NS P < 0.001
	1 g%	35.65 ± 10.45	P < 0.001		47.58 ± 8.67	P < 0.001		-1.56 ± 0.55	NS
Macerate extract	0.25 g%	16.20 ± 2.67	NS	NS	25.53 ± 1.67	P < 0.05	NS	-2.50 ± 0.80	NS P < 0.001
	0.5 g%	21.50 ± 4.26	P < 0.05	NS	41.06 ± 5.98	P < 0.001	NS	-1.70 ± 0.65	NS P < 0.001
	1 g%	28.80 ± 5.26	P < 0.001		52.67 ± 8.07	P < 0.001		-1.65 ± 0.7	NS

Values are presented as percentage change in proportion to maximum contraction obtained due to contractile agents and quoted as mean ± SEM. Group 1: experiments on tracheal chains contracted by 10 µM methacholine in the presence of ordinary Krebs solution. Group 2: experiments on tracheal chains contracted by 10 µM methacholine in the presence of calcium free Krebs solution. Group 3: experiments on tracheal chains contracted by 60 mM KCl in the presence of ordinary Krebs solution (for each group, n = 6). St. Dif: Statistical difference, NS: non significant difference, S: saline, D: diltiazem.

**Table 2: Correlation between the inhibitory effects of extracts from *Nigella sativa* with concentrations in groups 2 and 3 experiments.**

Experimental Groups	Aqueous extract		Macerated extract	
	R	p value	R	P value
Group 1	0.49	p < 0.05	0.48	P < 0.05
Group 2	0.558	p < 0.05	0.62	P < 0.005

extracts in group 3 experiments may suggest the absence of any effects on calcium channels and/or existence of an opening effect of potassium channels for these extracts from *Nigella sativa*. However, multiple substances may present in the extracts leading to its pharmacological effects. The extracts may contain one or more substances affecting calcium channels, but their effects are masked by other substances. Therefore, the calcium channel inhibitory effects of different fractions should be tested in future studies.

Although both aqueous and macerated extracts are aqueous in nature, the methods of extraction for the two extracts are different. For preparing aqueous extract the seed should be exposed to water steam (100°C) for 18–24 h. But for macerated extract it should be macerated with water at room temperature for 48 h while shaking intermittently. Therefore the ingredients of two extracts may be different. In aqueous extract more substances may be extracted, but some ingredients are destroyed due to the high temperature. However, the effect of two extract was not statistically different.

The cause of a small contraction effect of saline in all 3 groups and extracts in group 3 is presumably the continuous effects of the contractile substances (methacholine or KCl). Therefore, whenever extracts and diltiazem show inhibitory properties, they also inhibit this slight concentration effect.

The results of our previous studies [11,12] and present study suggest that anticholinergic, histamine H<sub>1</sub> inhibitory, and probable potassium channel opening effects of *Nigella Sativa* may contribute to the bronchodilatory effect of this plant. The other possible mechanisms responsible for the bronchodilatory effect of *Nigella sativa* are as follow: stimulation of β-adrenergic receptors [20], stimulation of inhibitory non-adrenergic non-cholinergic nervous system (NANC) or inhibition of stimulatory NANC [21], methylxanthine activity of the plant [22], and inhibition of phosphodiesterase [23]. The contribution of these mechanisms and the importance of those seen in our studies in the bronchodilatory effects of *Nigella sativa* should be clarified in further studies. In the present study only direct *in vitro* effective concentrations are shown and examined. Therefore to discover the applied effective con-

centrations or dosages of the extract more *in vivo* studies are required which is clearly dependent upon the administration route. In addition, the fractionation of the extracts and study of effective fraction are also needed for this purpose.

In conclusion, the results of the present study show that although the extracts of *Nigella sativa* showed inhibitory effects on pre-contracted tracheal chains in the presence of both ordinary and calcium free Krebs solutions, the absence of inhibitory effect of the extracts on KCl induced contraction of tracheal chains suggest that aqueous and macerated extracts have no inhibitory effect on calcium channels. Therefore, the calcium channel blocking effect of this plant dose not contribute to the relaxant effect of this plant on tracheal chains of guinea pigs. However, the results of this study suggested a potassium channel opening effect for the extracts from *Nigella sativa* that may contribute on the relaxant effect of this plant on tracheal chains of guinea pigs.

## Material and Methods

### Plant and extracts

*Nigella sativa* was collected from Torbat Heydarieh (north east Iran) in the spring of 2002, and dried at room temperature in the absence of sunlight. The plant was identified by botanists in the herbarium of Ferdowsi University of Mashhad; and the specimen number of the plant is 293-0303-1 *Nigella sativa*

The aqueous extract was prepared as follows: Fifty grams of the chopped, dried plant was extracted with 300 ml distilled water by suxhelat apparatus. Three hundred millilitres distilled water were added to a glass balloon in the lower part of suxhelat apparatus which was over a heater. The heater was set to boil distilled water continuously. The water steam flow through the middle part of the apparatus contained plant powder. The steam was then cooled and converted to liquid as it passed through a tube in the upper portion of the suxhelat with a helix tube inside through which tap water flowed. By means of aside tube, then added to the balloon in the lower part. This procedure was continued for 18–24 h until the liquid water returning to balloon through the side tube became colourless. For macerated extract, the same amount of plant was macerated with 300 ml distilled water (on a shaker) for 48 hr. The solvent of both extracts were then removed under reduced pressure until the extract volumes reached 20 ml. Plant ingredient concentration in the final extracts was 10% W/W in both extracts. The amount of thymoquinone, thymohydroquinone and thymol in the essential oil of *Nigella sativa* is 0.53 %, 0.77 % and 0.91 % respectively [24]. However, there is no information regarding the amount of main constituents in aqueous and macer-

ated extracts of this plant but their amount in extracts seems to be lower than in the essential oil.

### Tissue preparations

Male guinea pigs (400–700 g) were killed by a blow on the neck and trachea removed. Each trachea was cut into 10 rings (each containing 2–3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain [25].

Tissue was then suspended in a 10 ml organ bath (organ bath 61300, Bio Science Palmer-Washington, Sheerness, Kent U.K.) containing Krebs-Henseleit solution of the following composition (mM): NaCl 120, NaHCO<sub>3</sub> 25, MgSO<sub>4</sub> 0.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, KCl 4.72 and dextrose 11.

The Krebs solution was maintained at 37°C and gassed with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. Tissue was suspended under an isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with Krebs solution every 15 min.

### Protocols

The inhibitory effect of three different concentrations of aqueous and macerated extracts from *Nigella sativa* (0.25 g%, 0.5 g%, and 1 g% from each extract) and two concentrations of diltiazem (1 and 5 µM) in comparison with negative control (saline, 1 ml) on calcium channels was examined. To produce three concentrations of each extract 0.25 ml, 0.25 ml, and 0.5 ml of 10 W/V concentrated extract and for diltiazem 0.1 and 0.4 ml of 10 mM concentrated solution were added on a 10 ml organ bath at 5 min intervals.

In each experiment the effect of three cumulative concentrations from each extract, two cumulative concentrations of diltiazem or saline on contracted tracheal smooth muscle were measured after exposing tissue to the solution for 10 min. A decrease in tone was considered as an inhibitory effect and expressed as positive percentage change in proportion to maximum contraction obtained due to contractile agents.

The inhibitory effects of different solutions were tested with three different experimental designs as follows:

1. On tracheal chains contracted by 10 µM methacholine hydrochloride in the presence of ordinary Krebs solution (Sigma Chemical Ltd UK), (group 1 experiments).
2. On tracheal chains contracted by 10 µM methacholine hydrochloride in the presence of calcium free Krebs solution (group 2 experiments).

3. On tracheal chains contracted by 60 mM KCl in the presence of ordinary Krebs solution (group 3 experiments).

The inhibitory effects in three groups of experiments were examined in three different series of tracheal chains (for all groups,  $n = 6$ ). All of the experiments were performed randomly with a 1 h resting period of tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution. In all experiments responses were recorded on a kymograph (ET8 G-Boulitt, Paris) and were measured after fixation. The study was approved by the ethical committee of Mashhad University of Medical Sciences.

### Statistical analysis

The data of the inhibitory effect of different experiments were expressed as mean  $\pm$  SEM. The data of inhibitory effects of different concentrations of extracts, diltiazem, and saline were compared using ANOVA test in each group. The effect of each concentration of extracts and diltiazem between three groups was also compared using ANOVA. The inhibitory effect of different extracts were related to the concentrations of the solutions using least square regression. Significance was accepted at  $p < 0.05$ .

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